Attorney Docket No.: 34433/US/4/AMP/SKS
Dorsey File No. 474930-00006

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Allan *et al.*

Application Serial No.: 10/722,843

Examiner: Not Yet Assigned

Filed: November 25, 2003

Art Unit: 1646

For: Peptides Which Target Tumor and Endothelial Cells, Compositions and Uses Thereof

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Mail Stop Petition
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PETITION TO ACCORD A FILING DATE UNDER 37 CFR § 1.53 (e)

Applicants respectfully Petition to Accord Filing Date under 37 CFR § 1.53 of November 25, 2003, for above-referenced utility application, the date of receipt of the application by the United States Patent and Trademark Office. The Notice to File Missing Parts of Non-Provisional Application mailed July 8, 2004 stated that pages 27-49 of the above-referenced application were missing.

Accompanying this Petition are a copy of the Notice to File Missing Parts, a copy of pages 27-49 of the above referenced application, a copy of the return post card with the USPTO stamp acknowledging receipt of 99 pages of Description, 17 pages Claims and 1 page of Abstract and the required fee under 37 CFR § 1.17(h). Applicants maintain that the above-referenced application was complete as filed and request refund of the fee under 37 CFR § 1.17(h).

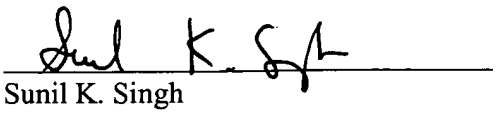
No fees beyond the petition fee under 37 CFR § 1.17(h) are believed due in connection with this Petition to Accord Filing Date. However, the Commissioner is authorized to charge any required fee not included with this Petition to Accord Filing Date or credit any overpayment to Dorsey & Whitney Deposit Account No. 50-2319 (Attorney Docket No. 34433/US/4/AMP/SKS, Dorsey File No. 474930-00006).

09/14/2004 HDEMESS1 00000047 502319 10722843
01 FC:1460 130.00 OP

Attorney Docket No.: 34433/US/4/AMP/SKS
Dorsey File No. 474930-00006

Date: September 7, 2004

Respectfully submitted,

By: 

Sunil K. Singh
Reg. No. 45,298

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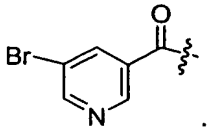
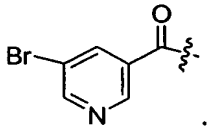
Intellectual Property Department
Suite 3400, Four Embarcadero Center
San Francisco, CA 94111-4187

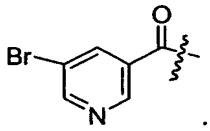
In still another embodiment of a compound of Formulae (I) and (II), r = 0, R^4 and R^5 are hydrogen, R^{14} is hydrogen, methyl or acetyl and R^1 is



In still another embodiment of a compound of Formulae (I) and (II), wherein r = 1, q = 2, R^4, R^5 and R^{25} are hydrogen, R^{14} is hydrogen, methyl or acetyl and R^1 is



In still another embodiment of a compound of Formulae (I) and (II), wherein r = 1, q = 2, R^1 is acetyl, R^4 and R^5 are hydrogen, R^{14} is hydrogen, methyl or acetyl and R^{25} is



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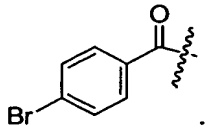

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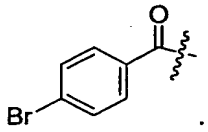
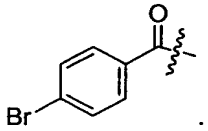


In still another embodiment of a compound of Formulae (I) and (II), r = 1, q = 2, R^1 is acetyl, R^4 and R^5 are hydrogen, R^{14} is hydrogen, methyl or acetyl and R^{25} is



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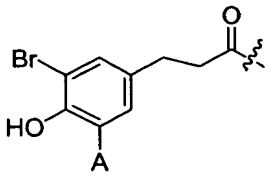

A is H or Br

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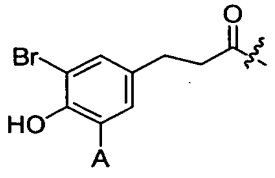

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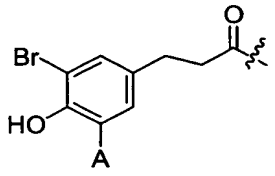

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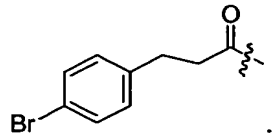

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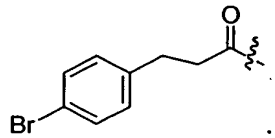

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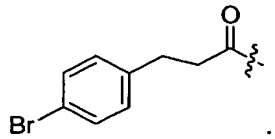

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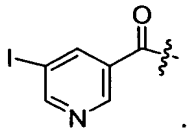

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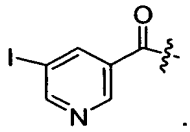

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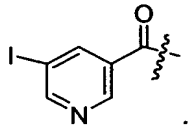

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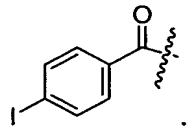

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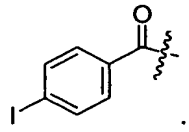

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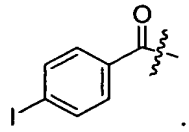

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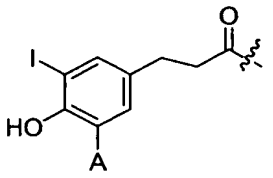

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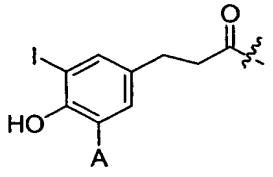

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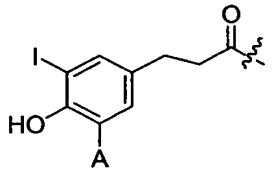

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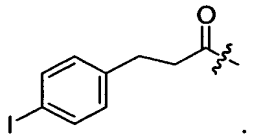

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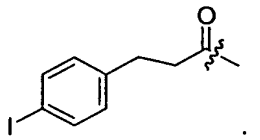

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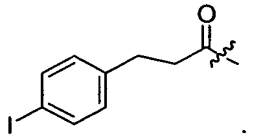

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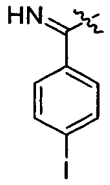

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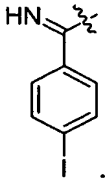

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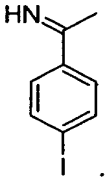

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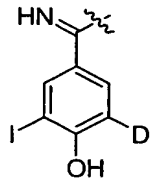

D = H or I

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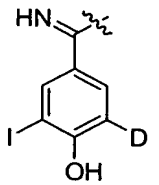

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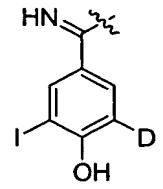
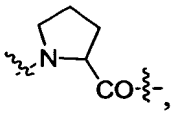
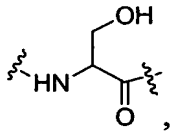


D = H or I

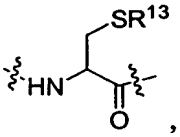
In a first preferred embodiment, R^1 is acyl or substituted acyl, R^2 is C_1-C_4 alkyl with at least one hydrogen atom replaced by a substituent selected from the group consisting of -NR^6R^7, aryl and substituted aryl, R^6 and R^7 are independently selected from the group consisting of hydrogen, acyl and substituted acyl, X_1 is -$NH(CH_2)_hCO$-, X_2 is



X_4 is

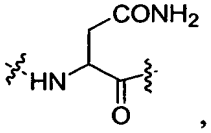


X_5 is



R^{13} is hydrogen, acyl, substituted acyl, alkyl or substituted alkyl.

X_6 is



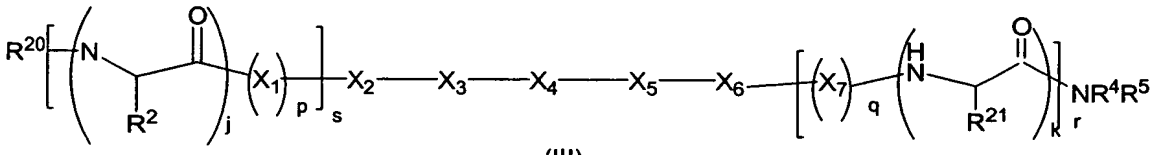
X_7 is -$NH(CH_2)_eCO$-, R^3 is C_1-C_4 alkyl with at least one hydrogen atom replaced by a substituent selected from the group consisting of -$NR^{15}R^{16}$, aryl and substituted aryl, R^{15} and R^{16} are independently selected from the group consisting of hydrogen, acyl and substituted acyl and R^4 and R^5 are hydrogen. In one embodiment, s is 0 and r is 1, k is 1, R^1 is acetyl, R^{13} is hydrogen, e is 1 and R^3 is $-(CH_2)_4NH_2$. Preferably, q is 2, 4 or 6. In another embodiment, s is 0 and r is 1, k is 1, R^1 is acetyl, R^{13} is hydrogen, e is

2, 4 or 6 and R^3 is $-(CH_2)_4NHCO(CH_2)_2$-Ph-(4-OH). Preferably, q is 1. In still another embodiment, s is 0 and r is 1, k is 1, R^1 is acetyl, R^{13} is hydrogen, e is 2, 4 or 6 and R^3 is -CH_2-Ph-(4-OH). Preferably, q is 1. In still another embodiment, s is 0 and r is 1, k is 1, R^1 is acetyl, R^{13} is methyl, e is 1 and R^3 is $-(CH_2)_4NH_2$. Preferably, q is 2. In still another embodiment, s is 1 and r is 0, j is 1, R^1 is acetyl, R^{13} is hydrogen, h is 1 and R^2 is -CH_2-Ph-(4-OH). Preferably, p is 2, 4 or 6. In still another embodiment, s is 1 and r is 0, j is 1, R^1 is acetyl, R^{13} is hydrogen, h is 2, 4, or 6 and R^2 is $-CH_2$-Ph-(4-OH). Preferably, p is 1. In still another embodiment, s is 1 and r is 0, j is 0, R^1 is -$CO(CH_2)_2$-Ph-(4-OH), R^{13} is hydrogen and h is 1. Preferably, p is 2, 4 or 6. In still another embodiment, s is 1 and r is 0, j is 0, R^1 is $-CO(CH_2)_2$-Ph-(4-OH), R^{13} is hydrogen and h is 2, 4 or 6. Preferably, p is 1. In still another embodiment, s is 0 and r is 0, R^1 is $-(CH_2)_2$-Ph-(4-OH) and R^{13} is hydrogen. In still another embodiment, s is 0 and r is 0, R^1 is -COPh-(4-F) and R^{13} is hydrogen. In still another embodiment, s is 0 and r is 1, k is 1, R^1 is acetyl, R^{13} is methyl or hydrogen, e is 1 and R^3 is $-(CH_2)_4NHCOPh$-(4-F). Preferably, q is 2. In still another embodiment, s is 0 and r is 1, k is 1, R^1 is acetyl, R^{13} is hydrogen, e is 1 and R^3 is -$(CH_2)_4$NH–8-[4'-fluorobenzylamino]suberoyl or -$(CH_2)_4NHCOCH_2F$. Preferably, q is 2. In still another embodiment, s is 1 and r is 0, j is 0, R^1 is 8-[4'-fluorobenzylamino]suberoyl or -$COCH_2F$, R^{13} is hydrogen and h is 2. Preferably, p is 1. In still another embodiment, s is 0 and r is 1, k is 1, R^1 is acetyl, R^{13} is hydrogen and R^3 is -CH_2Ph-(3-I, 4-OH) or -CH_2Ph-(3,5-diI, 4-OH). Preferably, q is 0. Preferably, q is 1 and e is 2. Preferably, q is 1 and e is 1. In still another embodiment, s is 1 and r is 0, j is 1, R^1 is acetyl, R^{13} is hydrogen and R^2 is -CH_2Ph-(3-I, 4-OH) or -CH_2Ph-(3,5-diI, 4-OH). Preferably, p is 0. In still another embodiment, s is 0 and r is 0, R^1 is $-CO(CH_2)_2$Ph (4-OH, 3, 5 di-I) and R^{13} is hydrogen. In still another embodiment, s is 1 and r is 0, j is 0, R^1 is $-CO(CH_2)_2$Ph (4-OH, 3, 5 di-I), h is 2 and R^{13} is hydrogen. Poreferably, p is 1. In still another embodiment, s is 1 and r is 0, j is 1, R^1 is acetyl, R^2 is -CH_2-Ph (4-OH, 3, 5 di-I), h is 2 and R^{13} is hydrogen. Preferably, p is 1. In still another embodiment, s is 0 and r is 1, R^3 is -$(CH_2)_4NHCO(CH_2)_2$-Ph (4-OH, 3, 5 di-I), e is 1 and R^{13} is hydrogen. Preferably, q is 2.

In still another embodiment, R^1 is acyl chelate, R^2, R^6, R^7, X_1, X_2, X_4, X_5, R^{13}, X_6, X_7, R^3, R^{15},R^{16}, R^4 and R^5 are as defined in the first embodiment. In one embodiment, s is 1 and r is 0, j is 0, R^1 is DOTA-In, h is 2 and R^{13} is hydrogen.

Preferably, p is 1. In another embodiment, s is 0 and r is 0, R^1 is DPTA or DPTA-In and R^{13} is hydrogen.

In another aspect, the present invention provides a compound of Formula (III):



(III)

or a pharmaceutically acceptable salt, solvate, hydrate or N-oxide thereof wherein:

R^{20} is acyl, substituted acyl, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, imino, substituted imino or a diagnostic agent;

R^{21} is C_1-C_6 alkyl with at least one hydrogen atom replaced by a substituent selected from the group consisting of $-NHR^{22}$;

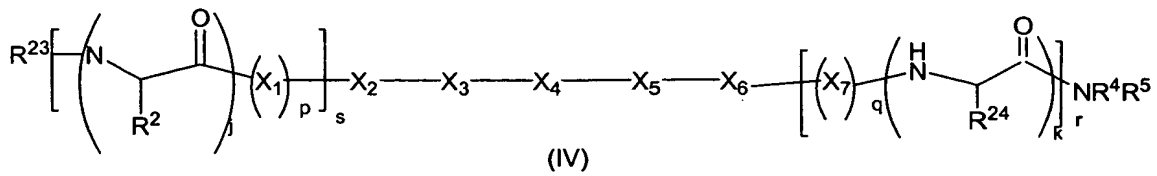
R^{22} is hydrogen, acyl, substituted acyl, alkyl, substituted alkyl or a diagnostic agent; and

j, k, p, q, r, s, R^2, X_1, X_2, X_3, X_4, X_5, X_6, X_7, R^4 and R^5 are as defined in structural formula (I);

with the proviso that at least one of R^{20} and R^{22} is a diagnostic agent.

In one embodiment, R^2, X_1, X_2, X_3, X_4, X_5, X_6, X_7, R^4 and R^5 are as defined in the first preferred embodiment. In one embodiment, R^{20} is a fluorescent agent. Preferably, R^{20} is 5/6 carboxy fluorescein, s is 1, r is 0, j is 0, e is 2 and p is 1. In another embodiment, R^{22} is a fluorescent agent. Preferably, R^{21} is $(CH_2)_4NH$-, R^{22} is-5/6 carboxy fluorescein, s is 0, r is 1, k is 1, e is 1 and q is 2. Preferably, R^{21} is $(CH_2)_4NH$-, R^{22} is biotin, s is 0, r is 1, k is 1, e is 1 and q is 2.

In anothere aspect, the present invention the present invention provides a compound of Formula (IV):



(IV)

or a pharmaceutically acceptable salt, solvate, hydrate or N-oxide thereof wherein:

R^{23} is acyl, substituted acyl, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, imino, substituted imino or a pegylating agent;

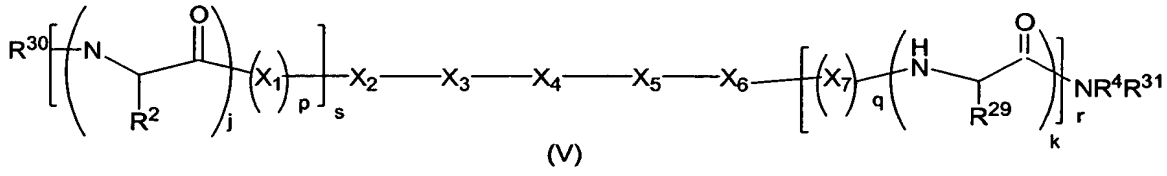
R^{24} is C_1-C_6 alkyl with at least one hydrogen atom replaced by a substituent selected from the group consisting of -NHR^{28} wherein R^{28} is hydrogen, acyl, substituted acyl, alkyl substituted alkyl or a pegylating agent; and

j, k, p, q, r, s, R^2, X_1, X_2, X_3, X_4, X_5, X_6, X_7, R^4 and R^5 are as defined in structural formula (I);

with the proviso that at least one of R^{23} or R^{28} is a pegylating agent.

In one embodiment, R^2, X_1, X_2, X_3, X_4, X_5, X_6, X_7, R^4 and R^5 are as defined in the first preferred embodiment. Preferably, R^{23} is m-dPEG, s is 1, r is 0, j is 0, h is 2 and p is 1.

In still another aspect , the present invention provides a compound of Formula (V):



(V)

or a pharmaceutically acceptable salt, solvate, hydrate or N-oxide thereof wherein:

R^{29} is C_1-C_6 alkyl with at least one hydrogen atom replace by–NHR^{32};

R^{30} is acyl, substituted acyl, alkyl, substituted alkyl or a therapeutic agent.

R^{31} is hydrogen, alkyl, substituted alkyl or a therapeutic agent;

R^{32} is hydrogen, acyl substituted acyl, alkyl, substituted alkyl or a therapeutic agent; and;

j, k, p, q, r, s, R^2, X_1, X_2, X_3, X_4, X_5, X_6, X_7 and R^4 and R^5 are as defined structural formula (I) ;

with the proviso that at least one of R^{30}, R^{31} and R^{32} is a therapeutic agent.

In one embodiment, R^2, X_1, X_2, X_3, X_4, X_5, X_6, X_7 and R^4 are as defined in the first preferred embodiment. Preferably, R^{13} is methyl or acetyl, s is 0, r is 0, R^{30} is acetyl and R^{31} is a therapeutic agent. In one embodiment, the therapeutic agent is doxorubicin. In another embodiment, R^{13} is methyl or hydrogen, s is 0, r is 1, k is 1, e is 1, q is 2, R^{30} is acetyl, R^{31} is hydrogen, R^{29} is $-(CH_2)_4NHR^{32}$. Preferably, R^{32} is $-CO(CH_2)_3$-doxorubicin or protoporphyrin.

The use of unnatural amino acids is specifically contemplated in the present invention. Accordingly, variations of compounds invention includes, for example, the D-amino acids of the naturally occurring amino acids, β-alanine, 3-aminopropionic acid, 2,3 diaminopropionic acid, 4-aminobutyric acid, *etc.*, sarcosine, orthinine, N-methyl glycine, citrulline, *t*-butyl alanine, homoarginine, *etc.* are within the scope of the present invention

One or amide bonds in the compounds of the invention may be optionally replaced by isosteres such as $-CH_2$-NH-, $-CH_2$-S-, $-CH_2$-S(O)-, $-CH_2$-$S(O)_2$-, $-COCH_2$- -CH=CH-, $CH(OH)CH_2$ which are well known in the art (see, *e.g.*, Spatola, "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," B. Weinstein, (eds.), Marcel Dekker, New York, 1983; Spatola *et al.*, *Life Sci.* 1986, 38:1243-1249; Almquist *et al.*, *J. Med. Chem.* **1980**, 23:1392; Holladay *et al.*, *Tetrahedron Lett.* **1983**, 24:4401; Hruby, *Life Sci.* 1982, 4; 189:199; Jennings-White *et al.*, *Tetrahedron Lett.* **1982**, 23:2533; Hruby, *Biopolymers* **1993**; *33*:1073-1082; Wiley *et al.*, *Med. Res. Rev.* **1993** *13*:327-384; Moore *et al.*, *Adv. in Pharmacol* **1995**, *33*:91-141; Giannis *et al.*, **1997**, *Adv. in Drug Res. 29*:1-78). The peptides of the invention may also contain

peptide mimetics such as those described in Olson *et al.*, *J. Med. Chem.* **1993**, 36:3039 and Chorev *et al.*, *Science* **1979**, 204:1210.

Covalent modifications of the compounds of the invention are within the scope of the current invention and may improve the solubility, absorption, biological half life, *etc.* Such modifications may be effected by selective reaction of specific amino acid residues with organic reagents. For example, histidine residues may be selectively reacted with diethylpyrocarbonate at pH 5.5-7 and *p*-bromophenacyl bromide at pH 6.0. Residues containing free amino groups may be selectively reacted with carboxylic acid anhydrides, imidoesters, pyridoxal phosphate, trinitrobenzenesulfonic acid, O-methylisourea, 2,4 pentanedione, glyoxylate, *etc.* Arginyl residues may be selectively reacted with phenylglyoxal, and various diones. Glutaminyl and asparaginyl residues may be deaminated under mildly acidic conditions to provide the corresponding glutamyl and aspartyl residues. Proline and lysine may be selectively hydroxylated while serine and threonine residues may be selectively phosphorylated. The α-amino groups of histidine and lysine may be selectively methylated (Creighton, *Proteins: Structure and Molecule Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)).

Derivatization with bi-functional cross-linking agents (*e.g.*, 1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-succinimide esters, esters of 4-azidosalicylic acid, homobifunctional imidoesters (*e.g.*, disuccinimidyl esters such as 3,3'- dithiobis(succinimidylpropionate)), bifunctional maleimides (*e.g.*, bis-N-maleimido-1,8-octane, *etc.*) may be used to link compounds with water-insoluble support matrices or other macromolecular carriers. Photoactivatable agents such as methyl-3-[(*p*-azidophenyl) dithio]propioimidate may also be used to attach compounds with water-insoluble support matrices. Alternatively, compounds may be directly reacted with reactive water-insoluble matrices (*e.g.*, cyanogen bromide-activated carbohydrates).

The present invention also includes longer peptides comprised of repeating units of the amino acid sequences of the compounds of the invention. In one embodiment, the repeating unit of such a multimer is the amino acid sequence of a compound where a, b, x, y, and z are 1. In another embodiment, the repeating unit is the amino acid sequence of a compound of invention where only one of a, b, x, y, and z is 0 and the rest are 1.

A multimer may be comprised of either the same or different combinations of repeating units comprised of amino acid sequences of compounds of structural formula (I). Such multimeric peptides can be made by either by chemical synthesis or by recombinant DNA techniques, followed by chemical modification of the cysteine residues. Preferably, the synthetic multimers have 2 to 12 repeats, more preferably, 2 to 8 repeats of the core peptide sequence. Accordingly, the total number of amino acids in the multimer should not exceed about 110 residues (or the equivalents, when including linkers or spacers).

A preferred multimer has the formula $P^1{}_n$ where P^1 is a pentapeptide, n is 2 to8. In another embodiment, a multimer has the formula $(P^1\text{-}X_m)_n\text{-}P^2$ where P^1 and P^2 are pentapeptides. P^1 and P^2 may be the same or different and each P^1 may represent a different pentapeptide derivative of structural formula (I). X is $C_1\text{-}C_5$ alkyl, $C_1\text{-}C_5$ polyether containing up to 4 oxygen atoms or Gly_z wherein, z = 1-6, m = 0 or 1 and n = 1-7.

A preferred recombinantly produced peptide multimer has the formula: $(P^1\text{-}Gly_z)_n\text{-}P^2$ where P^1 and P^2 are pentapeptides which are the same or different and each P^1 in the multimer may be a different pentapeptide, n = 1-100 and z = 0-6. The multimer may be optionally functionalized at both the N- and C-termini.

Compounds of the invention may be modified by the covalent attachment of any type of molecule as long as the modification does not prevent or inhibit biological function (*i.e.*, inhibition or prevention of angiogenesis, cell invasion, cell proliferation, *etc.*). For example, a compound of the invention may be modified by glycosylation, acetylation, pegylation, phosphorylation, amidation, proteolytic cleavage, linkage to cellular ligand or protein, *etc.* Preferably, compounds of the invention are conjugated to a therapeutic agent or a diagnostic agent either directly or through a linking moiety.

Preferably, the linking moiety is first attached to a diagnostic or therapeutic agent to form a linking moiety intermediate which is then further attached to a compound of structural formula (I). As will be apparent to the skilled artisan, the linking moiety can also be first attached to a compound of the invention to form a linking moiety intermediate which can then be attached to a diagnostic agent or therapeutic agent.

Typically, a linking moiety will include a linker and a linking group for conjugating a therapeutic agent or diagnostic agent to a peptide. The nature of the

linker will depend upon the particular application and the type of conjugation desired as the linker may be hydrophilic or hydrophobic, long or short, rigid or flexible. The linker may be optionally substituted with one ore more linking groups which may be either the same or different, accordingly providing polyvalent linking moieties which are capable of conjugating multiple therapeutic agents or diagnostic agents with a antibody.

A wide variety of linkers comprised of stable bonds suitable for spacing linking groups from the amino nitro compound are known in the art, and include by way of example and not limitation, alkyl, heteroalkyl, acyclic heteroatomic bridges, aryl, arylaryl, arylalkyl, heteroaryl, heteroaryl-heteroaryl, substituted heteroaryl-heteroaryl, heteroarylalkyl, heteroaryl-heteroalkyl and the like. Thus, the linker may include single, double, triple or aromatic carbon-carbon bonds, nitrogen-nitrogen bonds, carbon-nitrogen, carbon-oxygen bonds and/or carbon-sulfur bonds. Accordingly, functionalities such as carbonyls, ethers, thioethers, carboxamides, sulfonamides, ureas, urethanes, hydrazines, *etc.* may be included in a linker.

Choosing a suitable linker is within the capabilities of those of skill in the art. For example, where a rigid linker is desired, the linker may be rigid polyunsaturated alkyl or an aryl, biaryl, heteroaryl, *etc*. Where a flexible linker is desired, the linker may be a flexible peptide such as Gly-Gly-Gly or a flexible saturated alkanyl or heteroalkanyl. Hydrophilic linkers may be, for example, polyalcohols or polyethers such as polyalkyleneglycols. Hydrophobic linkers may be, for example, alkyls or aryls.

Preferably, a linking group is capable of mediating formation of a covalent bond with complementary reactive functionality of, for example, peptide to provide the therapeutic agent or diagnostic agent conjugated to the peptide. Accordingly, the linking group may be any reactive functional group known to those of skill in the art that will react with common chemical groups found in peptides (*e.g.*, amino, sulfhydryl, hydroxyl, carboxylate, imidizaloyl, guandinium, amide, *etc.*). Accordingly, the linking group may be, for example, a photochemically activated group, an electrochemically activated group, a free radical donor, a free radical acceptor, a nucleophilic group or an electrophilic group. However, those of skill in the art will recognize that a variety of functional groups which are typically unreactive under certain reaction conditions can be activated to become reactive.

Groups that can be activated to become reactive include, *e.g.*, alcohols, carboxylic acids and esters, including salts thereof.

The linking group may be $-NHR^1$, $-NH_2$, -OH, -SH, halogen, -CHO, $-R^1CO$, $-SO_2H$, $-PO_2H$, $-N_3$, -CN, $-CO_2H$, $-SO_3H$, $-PO_3H$, $-PO_2(O\ R^1)H$, $-CO_2R^1$, $-SO_3R^1$ or $-PO(OR^1)_2$ where R^1 is alkyl. Preferably, the linking group is $-NHR^1$, $-NH_2$, -OH, -SH, -CHO, $-CO_2H$, R^1CO-, halogen and-CO_2R^1.

Some embodiments of the linker and the linking group include, for example, compounds where the linker is $-(CH_2)_n-$, n is an integer between 1 and 8, the linking group is-NH_2, -OH, $-CO_2H$, and $-CO_2R^1$and the corresponding analogues where any suitable hydrogen is substituted. Other embodiments of the linking moiety include any amino acid, which may be, for example, a D or L amino acid. Thus, the linking moiety may be a dipeptide, a tripeptide or a tetrapeptide comprised of any combination of amino acids. The polarity of the peptide bond in these peptides may be either C-N or N-C.

Therapeutic agents and diagnostic agents may be linked to peptides directly using a variety of conventional reactions known to the skilled artisan. For example, condensation reagents (*e.g.*, carbodiimides, carbonyldiimidazoles, *etc.*) may be used to form an amide bond linkage between an amino group of the therapeutic or diagnostic agent and the carboxylic acid groups of residues such as glutamic acid, aspartic acid and the C-terminal carboxyl group of a compound of structural formula (I).

Similar methods may be used to attach therapeutic agents and diagnostic agents containing a linker and linking group to compounds of structural formula (I). For example, diagnostic agents and therapeutic agents containing a linker and linking group may be attached to the amino group of lysine, the carboxylic acid groups of glutamic acid and aspartic acid, the sulfhydryl group of cysteine, the hydroxyl groups of threonine and serine and the various moieties of aromatic amino acids of peptides using conventional approaches known to the skilled artisan. In general, selection of an appropriate strategy for conjugating diagnostic agents or therapeutic agents to a peptide either directly or through a linker and linking group is well within the ambit of the skilled artisan.

Therapeutic agents which can be conjugated to peptides include, but are not limited to, radionuclides, porphyrins and porphyrin derivatives for photodynamic

therapy (*e.g.*, protoporphyrin, benzoporphyrin derivative monoacid A, tin-etio purpurin, *meta*-tetrahydroxyphenylchlorin, HPD, photofrin, protoporhyrin IX, Pc4, mono aspartyl chlorin e_6, for others see T. Hassan *et al*, "PhotoDynamic Therapy of Cancer" in Cancer medicine, fifth edition, R.C. Blast *et al.*, Ed., B. C. Decker Inc, Canada, 2000, p. 489-502), protein toxins (*e.g.*, ricin, *Pseudomonas* exotoxin, diptheria toxin, saporin, pokeweed antiviral protein, bouganin, *etc.*), cytotoxic cancer agents, camptothecins (*e.g.*, 9-nitrocamptothecin (9NC), 9-aminocamptothecin (9AC), 10-aminocamptothecin, 9-chlorocamptothecin, 10,11-methylendioxycamptothecin, irinothecin, aromatic camptothecin esters, alkyl camptothecin esters, topotecan, (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione methanesulfonate dihydrate (DX-8951f), 7-[(2-trimethyl-silyl)ethyl]-20(S)camptothecin (BNP1350), Rubitecan, Exatecan, Lurtotecan, Diflomotecan and other homocamptothecins, *etc.*), taxanes (*e.g.*, taxol), epithilones, calicheamycins, hydroxy urea, cytarabine, cyclophosamide, ifosamide, nitrosureas, cisplatin, mitomycins maytansines, carboplatin, dacarbazine, procarbazine, etoposides, tenoposide, bleomycin, doxurobicin, 2-pyrrolinodoxurobicin, daunomycin, idarubican, daunorubicin, dactinomycin, plicamycin, mitoxantrone, asparginase, dihydroxy anthracine dione, mithrimycin, actinomycin D, 1-dehydrotestosterone, cytochlasins, vinblastine, vincristine, vinorelbine, paclitaxel, docetaxel, gramicidin D, glucocorticoids, anthracyclines, procaine, teracaine, lidocaine, propanolol, puromycin, methotrexate, 6-mercaptopurine, 6-thioguanine, mustard toxins, anthyrimycin, paclitaxel, alkylating agents (*e.g.*, mechoremethamine, thioepa chlorambucil, melphalan, carmustine, loustine, cyclothosphamide, busulfan, dibromomannitol, streptozotocin, *etc.*) homologues and analogues thereof. Preferably, the therapeutic agent is a cytotoxic cancer agent, such as, for example, a taxane, a camptothecin, an epithilone or a anthracycline. In one embodiment, the therapeutic agent is doxorubicin. In another embodiment the therapeutic agent is a radionuclide.

Also within the current invention is conjugation of the compounds with various peglyating agents. Representative peglyating agents include, but are not limited to, a-methoxy-w-carboxy-PEG 2K & 5K1, a-methoxy-w-N-succinimidylglutarate-PEG 2K & 5K1, a-methoxy-w-glutarate-PEG 2K, 5K, 20K, 30K2, a-methoxy-w-GGGglutarate-PEG 2K & 5K1, mPEG-Succinimidyl propionate 2K, 5K, 20K, 30K2 and m-PEG-ButyrALD 2K,5K, 20K, 30K2 (for other peglyating

agents see Li *et al.*, *Biomacromolecules*, **2003**, *4*, 1055.1067). Common pegylating agents are also available from commercial supplies such as Nektar Therapeutics, San Carlos, CA. Methods for attachment of various PEG groups to peptides are numerous and are well known to the skilled artisan.

The term "diagnostically labeled" means that a peptide has an attached diagnostically detectable label. Many different labels exist in the art and methods of labeling are well known the skilled artisan. General classes of labels, which can be used in the present invention, include but are not limited to, radioactive isotopes, paramagnetic isotopes, compounds which can be imaged by positron emission tomography (PET), fluorescent or colored compounds, compounds which can be imaged by magnetic resonance, chemiluminescent compounds, bioluminescent compounds, *etc.* Suitable detectable labels include, but are not limited to, radioactive, fluorescent, fluorogenic or chromogenic labels. Useful radiolabels (radionuclides), which are detected simply by gamma counter, scintillation counter or autoradiography include, but are not limited to, ^{3}H, ^{125}I, ^{131}I, ^{35}S and ^{14}C.

Methods and compositions for complexing metals to peptides are well known in the art. The metals are preferably detectable metal atoms, including radionuclides, and are complexed to proteins and other molecules (See, *e.g.*, U. S. Patent Nos. 5,627,286, 5,618,513, 5,567,408, 5,443,816 and 5,561,220).

Common fluorescent labels include, but are not limited to, fluorescein, rhodamine, dansyl, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine (Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, Sixth Ed., Molecular Probes, Eugene, OR, 1996) may be used to label compounds of structural formula (I). Fluorescein, fluorescein derivatives and fluorescein-like molecules such as Oregon Green™ and its derivatives, Rhodamine Green™ and Rhodol Green™, are coupled to amine groups using the isothiocyanate, succinimidyl ester or dichlorotriazinyl-reactive groups. Similarly, fluorophores may also be coupled to thiols using maleimide, iodoacetamide, and aziridine-reactive groups. The long wavelength rhodamines, which are basically Rhodamine Green™ derivatives with substituents on the nitrogens are preferred labeling reagents. This group includes the tetramethylrhodamines, X-rhodamines and Texas Red™ derivatives. Other preferred fluorophores are those excited by ultraviolet light. Examples include, but are not limited to, cascade blue, coumarin derivatives,

naphthalenes (of which dansyl chloride is a member), pyrenes and pyridyloxazole derivatives.

Inorganic materials such as semiconductor nanocrystals (Bruchez, *et al.*, **1998**, *Science 281*:2013-2016) and quantum dots, *e.g.*, zinc-sulfide-capped Cd selenide (Chan, *et al.*, *Science* **1998**, 281:2016-2018) may also be used as diagnostic labels.

Peptides can also be labeled with fluorescence-emitting metals such as ^{152}Eu or others of the lanthanide series. These metals can be attached to compounds of structural formula (I) through acyl chelating groups such as diethylenetriaminepentaacetic acid (DTPA), ethylene-diamine-tetraacetic acid (EDTA), *etc.*.

Radionuclides may be attached to peptides either directly or indirectly using an acyl chelating group such as DTPA and EDTA for *in vivo* diagnosis. The chemistry of chelation is well known in the art and varying ranges of chelating agent to peptide may be used to provide the labeled peptide. Of course, the labeled peptide must retain the biological activity of the native peptide.

Any radionuclide having diagnostic or therapeutic value can be used as the radiolabel in the present invention. In a preferred embodiment, the radionuclide is a γ -emitting or beta -emitting radionuclide, for example, one selected from the lanthanide or actinide series of the elements. Positron-emitting radionuclides, *e.g.* ^{68}Ga or ^{64}Cu, may also be used. Suitable gamma -emitting radionuclides include those which are useful in diagnostic imaging applications. The gamma -emitting radionuclides preferably have a half-life of from 1 hour to 40 days, preferably from 12 hours to 3 days. Examples of suitable gamma -emitting radionuclides include ^{67}Ga, ^{111}In, ^{99m}Tc, ^{169}Yb and ^{186}Re. Most preferably, the radionuclide is ^{99m}Tc.

Examples of preferred radionuclides (ordered by atomic number) are ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, ^{90}Y, ^{97}Ru, ^{99}Tc, ^{111}In, ^{123}I, ^{125}I, ^{131}I, ^{169}Yb, ^{186}Re, and ^{201}Tl. Though limited work have been done with positron-emitting radiometals as labels, certain proteins, such as transferrin and human serum albumin, have been labeled with ^{68}Ga.

A number of metals (not radioisotopes) useful for magnetic resonance imaging include gadolinium, manganese, copper, iron, gold and europium. Gadolinium is most preferred. Generally, the amount of labeled peptide needed for detectability in diagnostic use will vary depending on considerations such as age, condition, sex, and

extent of disease in the patient, contraindications, if any, and other variables, and is to be adjusted by the individual physician or diagnostician. Dosage can vary from 0.01 mg/kg to 100 mg/kg.

Peptides may also be detected by coupling to a phosphorescent or a chemiluminescent compound, as is well known to the skilled artisan. Preferred chemiluminescent compounds include but are not limited to, luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Similarly, bioluminescent compounds may be used to detect antibodies and/or conjugates thereof and include, but are not limited to, luciferin, luciferase and aequorin.

Colorimetric detection, based on chromogenic compounds which have, or result in, chromophores with high extinction coefficients may also be used to detect compounds of structural formula (I).

4.3 Synthesis

The compounds of the invention may be obtained *via* conventional synthetic methods. Starting materials useful for preparing compounds of the invention and intermediates thereof are commercially available or can be prepared by well-known synthetic methods.

Peptides may be prepared using solid-phase synthesis such as that generally described by Merrifield, *J. Amer. Chem. Soc.* **1963**, *85*:2149-54 using automated equipment, which may be purchased from chemical suppliers (*e.g.*, Applied Biosystems, Foster City, CA) or manual equipment. Solid-phase peptide synthesis may be initiated from the C-terminus of the peptide by coupling a protected α-amino acid (either Boc or FMOC protected), to a suitable resin. Such a starting material can be prepared by attaching an α-amino-protected amino acid by an ester linkage to a chloromethylated resin, hydroxymethyl resin, BHA resin, MBHA resin or a Rink resin. Such methods, well-known in the art, are disclosed, for example, in United States Patent No. 5,994,309. Alternatively, compounds of the invention may be made by solution phase synthesis using protected α-amino acids (see *e.g.*, Bodanszky, "Methods of Peptide Synthesis," Springer Verlag, New York, 1984). As is apparent to those of skill in the art, unnatural amino acids can be easily employed in the above standard methods of chemical synthesis and may be made by conventional methods know to those of skill in the art.

The skilled artisan will appreciate that two general synthetic strategies exist for synthesis of compounds of the invention. Compounds with sulfur containing amino acids may be synthesized either directly by incorporation of the appropriate sulfur containing amino acid into a standard method of chemical synthesis as described above or indirectly by selective functionalization of an appropriate thiol containing peptide precursor and, if necessary, selective oxidation of the resultant thioether containing peptide. Methods for selectively functionalizing free thiols (*e.g.*, selective alkylation, acylation, disulfide formation, *etc.*) in the presence of diverse organic functionality are well known to the skilled artisan as are methods of oxidizing sulfides to sulfoxides (*e.g.*, $NaBO_3$, acetonitrile: water, $NaIO_4$, acetonitrile: water, *etc.*) and sulfones (*e.g.*, H_2O_2, HCO_2H).

4.4 Assays for Compounds of the Invention

Those of skill in the art will appreciate that the *in vitro* and *in vivo* assays useful for measuring the activity of the compounds of the invention described herein are illustrative rather than comprehensive.

4.4.1 Assay for endothelial cell migration

For endothelial cell (EC) migration, transwells are coated with type I collagen (50 μg/mL) by adding 200 μL of the collagen solution per transwell, then incubating overnight at 37°C. The transwells are assembled in a 24-well plate and a chemoattractant (*e.g.*, FGF-2) is added to the bottom chamber in a total volume of 0.8 mL media. ECs, such as human umbilical vein endothelial cells (HUVEC), which have been detached from monolayer culture using trypsin, are diluted to a final concentration of about 10^6 cells/mL with serum-free media and 0.2 mL of this cell suspension is added to the upper chamber of each transwell. Inhibitors to be tested may be added to both the upper and lower chambers and the migration is allowed to proceed for 5 hrs in a humidified atmosphere at 37°C. The transwells are removed from the plate stained using DiffQuik®. Cells which did not migrate are removed from the upper chamber by scraping with a cotton swab and the membranes are detached, mounted on slides, and counted under a high-power field (400x) to determine the number of cells migrated.

4.4.2 Biological Assay of Anti-Invasive Activity

The ability of cells such as ECs or tumor cells (*e.g.*, PC-3 human prostatic carcinoma) cells to invade through a reconstituted basement membrane (Matrigel®) in an assay known as a Matrigel® invasion assay system has been described in detail in the art (Kleinman *et al.*, *Biochemistry* **1986**, *25*: 312-318; Parish *et al.*, **1992**, *Int. J. Cancer 52*:378-383). Matrigel® is a reconstituted basement membrane containing type IV collagen, laminin, heparan sulfate proteoglycans such as perlecan, which bind to and localize bFGF, vitronectin as well as transforming growth factor-β (TGFβ), urokinase-type plasminogen activator (uPA), tissue plasminogens activator (tPA) and the serpin known as plasminogen activator inhibitor type 1 (PAI-1) (Chambers *et al.*, *Canc. Res.* **1995**, *55*:1578-1585). It is accepted in the art that results obtained in this assay for compounds which target extracellular receptors or enzymes are predictive of the efficacy of these compounds *in vivo* (Rabbani *et al.*, *Int. J. Cancer* **1995**, *63*: 840-845).

Such assays employ transwell tissue culture inserts. Invasive cells are defined as cells which are able to traverse through the Matrigel® and upper aspect of a polycarbonate membrane and adhere to the bottom of the membrane. Transwells (Costar) containing polycarbonate membranes (8.0 μm pore size) are coated with Matrigel® (Collaborative Research), which has been diluted in sterile PBS to a final concentration of 75 μg/mL (60 μL of diluted Matrigel® per insert), and placed in the wells of a 24-well plate. The membranes are dried overnight in a biological safety cabinet, then rehydrated by adding 100 μL of DMEM containing antibiotics for 1 hour on a shaker table. The DMEM is removed from each insert by aspiration and 0.8 mL of DMEM/10 % FBS/antibiotics is added to each well of the 24-well plate such that it surrounds the outside of the transwell ("lower chamber"). Fresh DMEM/ antibiotics (100μL), human Glu-plasminogen (5 μg/mL), and any inhibitors to be tested are added to the top, inside of the transwell ("upper chamber"). The cells which are to be tested are trypsinized and resuspended in DMEM/antibiotics, then added to the top chamber of the transwell at a final concentration of 800,000 cells/mL. The final volume of the upper chamber is adjusted to 200 μL. The assembled plate is then incubated in a humid 5% CO_2 atmosphere for 72 hours. After incubation, the cells are fixed and stained using DiffQuik® (Giemsa stain) and the upper chamber is then scraped using a cotton swab to remove the Matrigel® and any cells which did not

invade through the membrane. The membranes are detached from the transwell using an X-acto® blade, mounted on slides using Permount® and cover-slips, then counted under a high-powered (400x) field. An average of the cells invaded is determined from 5-10 fields counted and plotted as a function of inhibitor concentration.

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4.4.3 Tube-Formation Assays of Anti-Angiogenic Activity

Endothelial cells, for example, human umbilical vein endothelial cells (HUVEC) or human microvascular endothelial cells (HMVEC) which can be prepared or obtained commercially, are mixed at a concentration of 2×10^5 cells/mL with fibrinogen (5mg/mL in phosphate buffered saline (PBS) in a 1:1 (v/v) ratio. Thrombin is added (5 units/ mL final concentration) and the mixture is immediately transferred to a 24-well plate (0.5 mL per well). The fibrin gel is allowed to form and then VEGF and bFGF are added to the wells (each at 5 ng/mL final concentration) along with the test compound. The cells are incubated at 37°C in 5% CO_2 for 4 days at which time the cells in each well are counted and classified as either rounded, elongated with no branches, elongated with one branch, or elongated with 2 or more branches. Results are expressed as the average of 5 different wells for each concentration of compound. Typically, in the presence of angiogenic inhibitors, cells remain either rounded or form undifferentiated tubes (*e.g.* 0 or 1 branch). This assay is recognized in the art to be predictive of angiogenic (or anti-angiogenic) efficacy *in vivo* (Min *et al.*, *Cancer Res.* **1996**, *56*: 2428-2433).

In an alternate assay, endothelial cell tube formation is observed when endothelial cells are cultured on Matrigel® (Schnaper *et al.*, *J. Cell. Physiol.* **1995**, *165*:107-118). Endothelial cells (1×10^4 cells/well) are transferred onto Matrigel®-coated 24-well plates and tube formation is quantitated after 48 hrs. Inhibitors are tested by adding them either at the same time as the endothelial cells or at various time points thereafter. Tube formation can also be stimulated by adding (a) angiogenic growth factors such as bFGF or VEGF, (b) differentiation stimulating agents (*e.g.*, PMA) or (c) a combination of these.

While not wishing to be bound by theory, this assay models angiogenesis by presenting to the endothelial cells a particular type of basement membrane, namely the layer of matrix which migrating and differentiating endothelial cells might be expected to first encounter. In addition to bound growth factors, the matrix components found in

Matrigel® (and in basement membranes *in situ*) or proteolytic products thereof may also be stimulatory for endothelial cell tube formation which makes this model complementary to the fibrin gel angiogenesis model previously described (Blood *et al.*, *Biochim. Biophys. Acta* **1990**, *1032*:89-118; Odedra*t al.*, *Pharmac. Ther.* **1991**, *49*:111-124).

4.4.4. Assays for Inhibition of Proliferation

The ability of the compounds of the invention to inhibit the proliferation of EC's may be determined in a 96-well format. Type I collagen (gelatin) is used to coat the wells of the plate (0.1-1 mg/mL in PBS, 0.1 mL per well for 30 minutes at room temperature). After washing the plate (3x w/PBS), 3-6,000 cells are plated per well and allowed to attach for 4 hrs (37 ^{0}C/5% CO_2) in Endothelial Growth Medium (EGM; Clonetics) or M199 media containing 0.1-2% FBS. The media and any unattached cells are removed at the end of 4 hrs and fresh media containing bFGF (1-10 ng/mL) or VEGF (1-10 ng/mL) is added to each well. Compounds to be tested are added last and the plate is allowed to incubate (37 ^{0}C/5% CO_2) for 24-48 hrs. MTS (Promega) is added to each well and allowed to incubate from 1-4 hrs. The absorbance at 490nm, which is proportional to the cell number, is then measured to determine the differences in proliferation between control wells and those containing test compounds.

A similar assay system can be set up with cultured adherent tumor cells. However, collagen may be omitted in this format. Tumor cells (*e.g.*, 3,000-10,000/well) are plated and allowed to attach overnight. Serum free medium is then added to the wells,, and the cells are synchronized for 24 hrs. Medium containing 10% FBS is then added to each well to stimulate proliferation. Compounds to be tested are included in some of the wells. After 24 hrs, MTS is added to the plate and the assay developed and read as described above.

4.4.5 Assays of Cytotoxicity

The anti-proliferative and cytotoxic effects of compounds of the invention may be determined for various cell types including tumor cells, ECs, fibroblasts and macrophages. This is especially useful when testing a compound of the invention which has been conjugated to a therapeutic moiety such as a radiotherapeutic or a toxin. For example, a conjugate of one of the compounds of the invention with Bolton-Hunter reagent which has been iodinated with ^{131}I would be expected to

inhibit the proliferation of cells expressing an PHSCN binding site/receptor (most likely by inducing apoptosis). Anti-proliferative effects would be expected against tumor cells and stimulated endothelial cells but, under some circumstances not quiescent endothelial cells or normal human dermal fibroblasts. Any anti-proliferative or cytotoxic effects observed in the normal cells may represent non-specific toxicity of the conjugate.

A typical assay would involve plating cells at a density of 5-10,000 cells per well in a 96-well plate. The compound to be tested is added at a concentration 10x the IC_{50} measured in a binding assay (this will vary depending on the conjugate) and allowed to incubate with the cells for 30 minutes. The cells are washed 3X with media, then fresh media containing [^{3}H]thymidine (1 μCi/mL) is added to the cells and they are allowed to incubate at 37°C in 5% CO_2 for 24 and 48 hours. Cells are lysed at the various time points using 1 M NaOH and counts per well determined using a β–counter. Proliferation may be measured non-radioactively using MTS reagent or CyQuant® to measure total cell number. For cytotoxicity assays (measuring cell lysis), a Promega 96-well cytotoxicity kit is used. If there is evidence of anti-proliferative activity, induction of apoptosis may be measured using TumorTACS (Genzyme).

4.4.6 Caspase-3 Activity

The ability of the compounds of the invention to promote apoptosis of EC's may be determined by measuring activation of caspase-3. Type I collagen (gelatin) is used to coat a P100 plate and $5x10^5$ ECs are seeded in EGM containing 10% FBS. After 24 hours (at 37°C in5% CO_2) the medium is replaced by EGM containing 2% FBS, 10 ng/ml bFGF and the desired test compound. The cells are harvested after 6 hours, cell lysates prepared in 1% Triton and assayed using the EnzChek®Caspase-3 Assay Kit #1 (Molecular Probes) according to the manufactures' instructions.

4.4.7. Corneal Angiogenesis Model

The protocol used is essentially identical to that described by Volpert *et al.*, *J. Clin. Invest.* **1996**, *98*:671-679. Briefly, female Fischer rats (120-140 gms) are anesthetized and pellets (5 μl) comprised of Hydron®, bFGF (150 nM), and the